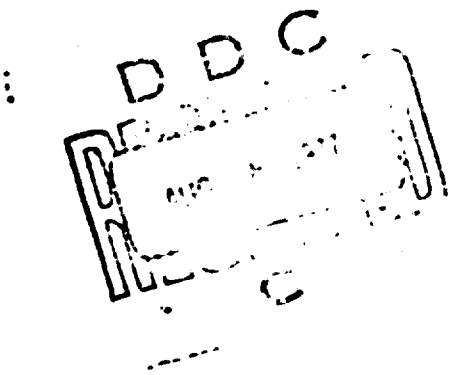
FINAL REPORT

on

TOXICOLOGICAL INVESTIGATIONS OF PILOT TREATMENT PLANT WASTEWATERS AT HOLSTON ARMY AMMUNITION PLANT

to

U.S. ARMY MEDICAL
RESEARCH AND DEVELOPMENT COMMAND



July 27, 1977

by

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EXECUTIVE SUMMARY

The United States Army Medical Research and Development Command has been supporting research in order to recommend environmental quality standards for the munitions manufacturing industry. Both laboratory and field studies have been conducted by a number of different contractors.

The present work conducted at the Holston Army Ammunition Plant in Kingsport, Tennessee was performed by Battelle's Columbus Laboratories in order to determine the toxicity of five wastewaters associated with a pilot biological treatment plant. Manufacturing wastewaters from both Area A and Area B were mixed in a ratio of 1:9 by volume, respectively. This mixture was then treated by two biological systems - the 3A system which consisted of an activated sludge chamber and the 6A system which contained both a trickling filter and an activated sludge chamber.

Work performed at HAAP included on-site 96-hour static acute LC_{50} bioassay tests using fathead minnows. Solutions tested were Area A wastes, Area B wastes, the A+B mixture, the 3A system effluent and the 6A system effluent. Quantitative analyses of RDX, HMX, TNT, and COD in the test waters were also conducted in conjunction with each test. Other water quality parameters monitored by HAAP personnel were correlated with munitions constituents and fish mortality. In-house Ames Spot tests were conducted on all wastewaters from the HAAP pilot plant.

Results of the bioassay tests with fathead minnows indicated the Area A wastewater to be the most toxic having an LC_{50} value of approximately 1 percent. Area B wastewater was found to be less toxic to this species but showed a greater daily fluctuation in toxicity with LC_{50} values ranging from 6.2 percent to 43.8 percent. The combined Area A + Area B wastes were intermediate in toxicity with LC_{50} values of 1.43 percent to 14.0 percent. Generally, the treated wastewaters (3A and 6A) were of lower toxicity with LC_{50} values of 70 percent or greater. Three replicate exceptions ranged between 16.5 percent and 36.7 percent.

A special bioassay was conducted using 6A effluent water spiked with 10 ppm RDX. Subsequent chemical analysis indicated the concentration of RDX in the test water to be 5.17 ppm. Results of this test produced an LC_{50} value of 69.8 percent.

Toxic effects (i.e., mortality) were positively correlated with high levels of HMX, COD, BOD (filtered and unfiltered), pH, NH_3, TKN, PO_4, NO_2, and total solids.

A review of the mutagenic screening test results of all wastewaters indicated that no definite mutagens were present. One sample of the 6A effluent collected on June 22, 1976, showed a slight suggestion of the possible presence of mutagens, however, the indication was marginal.

cont → The overall results of the on-site bioassay tests indicate that biological treatment, either activated sludge or the combination trickling-filter-activated sludge does reduce the toxicity of the HAAP manufacturing wastewaters.

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INTRODUCTION

Background

The United States Army Medical Research and Development Command (USAMRDC) has been supporting a major research effort, the ultimate objective of which is to develop suggested environmental quality standards for the munitions industry. Major efforts by several contractors have included both laboratory and field studies of the toxic effects of munitions manufacturing waste waters. Battelle's Columbus Laboratories (BCL) have been involved in field studies since 1974.

Phase I investigations involved screening studies at three munitions plants. These three plants were:

- Badger Army Ammunition Plant (BAAP)
- Joliet Army Ammunition Plant (JAAP)
- Lake City Army Ammunition Plant (LCAAP)

Results of Phase I investigations are described in Cooper et al., 1975.

Phase II studies concentrated on environmental effects at BAAP and JAAP. The objectives of Phase II investigations were to collect and analyze replicated quantitative information relative to the specific nature of effluent effects on receiving systems and to determine the relationship(s) between observed effects and the amount of primary munitions constituents in the effluents. Results of these investigations were reported in two separate reports to the USAMRDC for work at BAAP (Stilwell et al., 1976a) and at JAAP (Stilwell et al., 1976b).

Purpose

Under USAMRDC Contract No. DAMD 17-74-C-4123 BCL has been conducting additional related work at the Holston Army Ammunition Plant (HAAP). The purpose of these further investigations was to generate on-site toxicological data on the influents to and effluents from the waste treatment

pilot plant being tested at HAAP. This pilot plant has been developed and implemented by U.S. Army Armament Command (ARMCOM) to evaluate the effectiveness of advanced munitions waste treatment concepts and desensitization.

These investigations will allow for a more thorough evaluation of the overall effectiveness of the biological treatment concepts being tested.

Scope

The scope of the work performed at HAAP included the following:

- On-site 96-hour static acute LC_{50} bioassays using fathead minnows (Pimephales promelas). Solutions tested were Area A wastes, Area B wastes, 1:9 mixture of Area A and Area B, the activated sludge system effluent (3A) and the aerobic trickle filter - activated sludge system effluent (6A).
- Quantitative analyses of RDX, HMX, TNT and COD in all bioassay test waters.
- In-house Ames Spot tests on influent and effluent waste waters from the HAAP pilot plant.

RESEARCH STRATEGY

The main emphasis of current investigations at HAAP was on the determination of the toxicity of the five wastewaters selected for study. The relative toxicity of each solution was tested during multiple, duplicate bioassays conducted on-site in a mobile laboratory. Water samples were collected, preserved and analyzed by BCL and HAAP personnel in conjunction with each test. These studies were not designed to determine the fate of various munitions compounds or the efficiencies of the component parts of the treatment process. They were designed to measure only the toxic properties of the influents and effluents of the pilot treatment plant.

Sampling Protocol

Facility Description

Holston Army Ammunition Plant is a government-owned contractor-operated installation located in northeastern Tennessee in Kingsport. Operated by Eastman Corporation, its primary missions are the manufacture of RDX and HMX and the preparation and loading of Composition B (a mixture of RDX and TNT). The plant is located in two areas of Kingsport, Tennessee. Area A, located in Kingsport proper, occupies an area along the south fork of the Holston River. Area B is located southeast of Kingsport on the main fork of the Holston River downstream from the north and south fork confluence.

Manufacturing Waste Composition

The influent to the pilot biological treatment plant was composed of Area A waste and Area B waste mixed in a 1 to 9 ratio by volume to approximate actual discharges.

Table 1 presents the points where the Area A and Area B composite wastewaters were collected. Area A wastes were composed of ten different wastewaters. Area B was sampled at four points to form the explosives manufacturing area wastewater. An additional synthetic waste composed of acetic acid, cyclohexanone, acetone, n-butanol, and hexamine was added to the Area B composite to bring the COD level up to 400 mg/l, which more closely approximates actual manufacturing wastewater levels at high production periods. Nitrates were also added at the rate of 150 mg/l to simulate actual levels under high productive conditions.

Pilot Plant Description

The pilot waste treatment plant was constructed in building E-2 in the explosives manufacturing area. Wastewater flow through the pilot plant is depicted in the flow chart in Figure 1. A flow description follows:

TABLE 1. HOLSTON ARMY AMMUNITION PLANT MANUFACTURING WASTE COMPOSITION

Point	Wastewater	Volume (gallons)
	Area A	
Acetic Acid Concentration	Azeo Slop Water	91.0
Acetic Acid Concentration	Sludge Heater Waste	4.0
Acetic Acid Concentration	Azeo Slop Water	66.0[a]
Acetic Acid Concentration	Sludge Heater Waste	4.0[a]
Acetic Anhydride Refining	Low Boiler Waste	0.25
Acetic Anhydride Manufacturing	E-Scrubber	40.0
Steam Generation	Boiler Blowdown	47.5
Product Gas Plant	River Water (hairpin cooler)	230.0
Filter Plant	Lime and Alum Sludge	12.0
Filter Plant	Ion Exchange Regeneration	6.5
		501.25[b]
	Area B	
1	Composition B production line	50
2	Composition B production line	200
3	Continuous process RDX line	100
4	Acetic acid, NO_2/NO_3, laundry, $NaNO_3$ and other manufacturing wastes	150
		500

(a) Because of low production these samples were collected from the same building as the two previous wastewaters.

(b) Add 0.8 lb. of pulverized boiler bottom ash (can be from Area B).

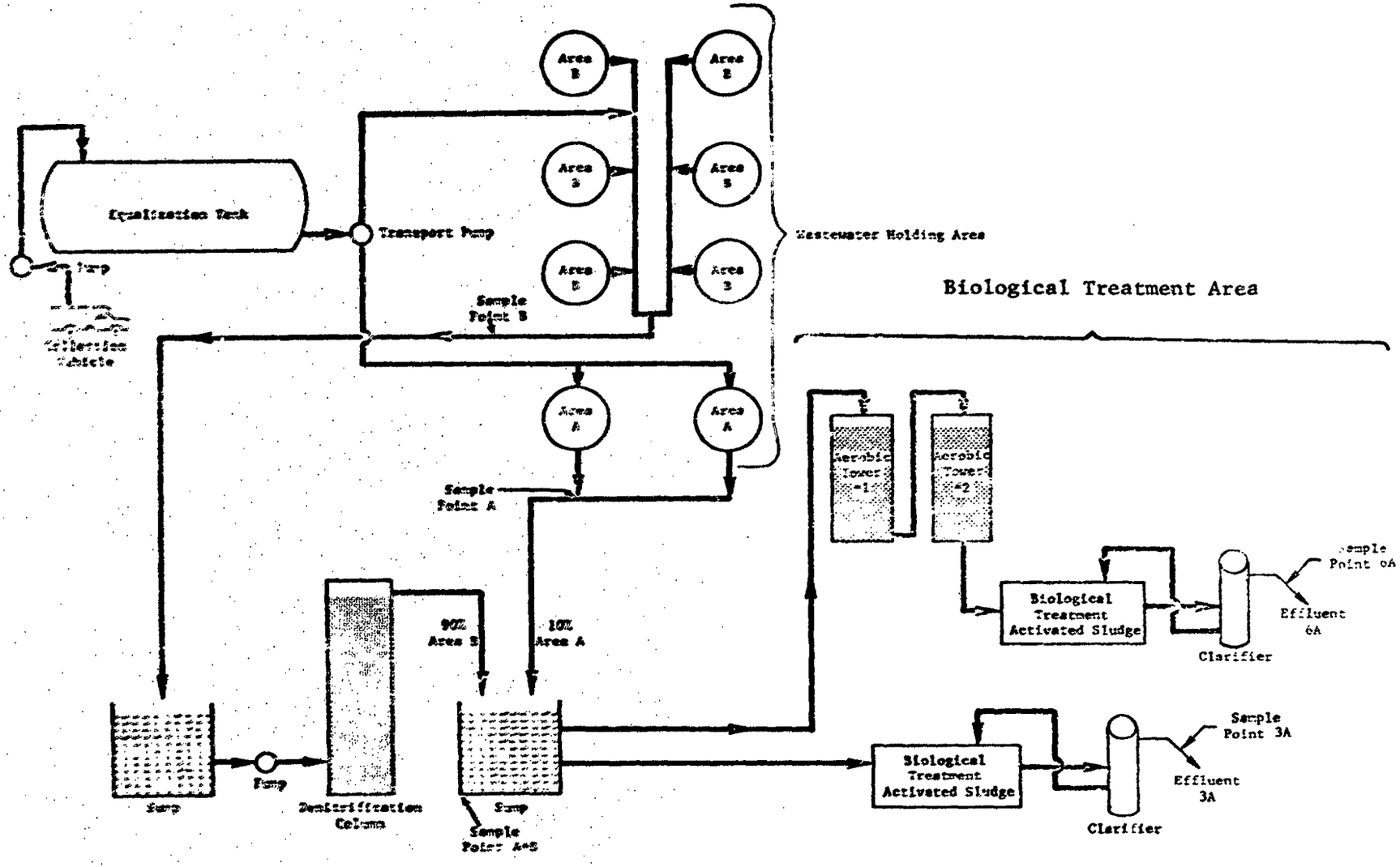


FIGURE 1. HAAP BIOLOGICAL TREATMENT PILOT PLANT FLOW DIAGRAM

- Area A and Area B wastes were collected separately and transported to building E-2 where each was pumped into the equalization tank. Each wastewater was pumped to separate storage containers inside the building.
- Synthetic wastes were added to the Area B wastes in the storage tanks to increase the COD to 400 mg/l and the nitrate-nitrogen to 150 mg/l.
- The Area B wastes were passed through a sump to a denitrification column. Nitrate reduction was usually greater than 90% through this column.
- The Area A and Area B wastes were mixed in a sump in the ratio of 1:9.
- The A and B mixture was fed into two separate biological treatment systems - 3A and 6A.

  3A System

  A and B blend was fed into an activated sludge chamber and finally to a clarifier. Settleable material was recirculated to the sludge chamber. The overflow from the clarifier constituted the final 3A effluent.

  6A System

  The A and B blend was pretreated through two aerobic trickle filter towers before being fed into an activated sludge chamber. The discharge from the sludge treatment war fed into a clarifier. As in the 3A system, settleable material in the clarifier was recirculated to the activated sludge. The overflow from the clarifier in this system constituted the 6A effluent.

Bioassay Test Sample Points

Bioassay test waters were collected from five points within the pilot plant system. These points are shown on Figure 1. The wastewater solutions and numbers of tests run are presented in Table 2.

TABLE 2. WASTEWATERS USED IN FISH BIOASSAY TESTS AT HAAP

Waste Water	Sampling Location	Number of Bioassay Tests
Area A	Holding Tank Drain	5
Area B	Holding Tank Drain	3
Area B (without COD or nitrate added)	Holding Tank Drain	1
Area A + Area B	Mixing Sump	3
Effluent 3A	Clarifier Overflow	5
Effluent 6A	Clarifier Overflow	7

Sampling and Analytical Methods

Sample Collection

Water samples from the five sample points in the pilot plant were collected daily in plastic bottles and analyzed in the plant chemistry laboratory by HAAP personnel. Samples were composited over 24-hours and kept on ice during the sampling period.

Water samples for the fish bioassay tests and BCL chemical analysis were collected by BCL personnel in 5-gallon glass bottles and transported to the mobile laboratory located near the HAAP pump house on the Holston River. Water samples for chemical analysis by BCL were taken from the bioassay water, preserved in amber glass bottles or plastic (COD analysis) and refrigerated on-site. Samples for mutagenic screening were taken from the bioassay water, put in plastic bottles and refrigerated. All BCL samples were then packed on ice and delivered to the laboratory in Columbus, Ohio.

Analytical Methods

HAAP Water Quality. Water samples were collected at points within the pilot plant and analyzed for selected water quality parameters by HAAP personnel. Analytical techniques for COD, BOD, TKN, dissolved oxygen, total phosphate, settleable solids and total solids were those detailed in "Standard Methods for the Examination of Water and Wastewater" the 13th edition (1971). Nitrate and nitrite were measured using Orion specific ion electrodes. Ammonia was measured as nitrogen by the Kjeldahl method and pH was done with a Foxboro recording pH meter. Munitions constituents including RDX, HMX, and TNT were measured using a liquid chromatography method.

Battelle's Columbus Laboratory Munitions Constituent Analysis. Methods for the determination of microgram and submicrogram quantities of HMX, RDX, and TNT are reported in the literature and include titrimetric (Sinha et al., 1964; Simecek, 1961; Fauth, 1965), thin layer chromatographic (Fauth and Roecker, 1965; Harthon, 1961; Glover and Hoffsommer, 1973; Bell

and Dunstan, 1966, and Hannson and Olin, 1962) as well as gas chromatographic (Hoffsommer, 1970; Glover and Hoffsommer, 1974; Hoffsommer, et al., 1975; Hoffsommer and Rosen, 1972; Rowe, 1967; Cooper et al., 1974) procedures. However these techniques all require somewhat laborious sample preparatory procedures including extraction and derivatization. These procedures introduce sources of serious errors resulting from sample loss and degradation, in addition to being time consuming and tedious. A method for the gas chromatographic analysis of TNT and 2,4- and 2,6-DNT in water and sediment samples had been developed at Battelle's Columbus Laboratories (Cooper et al., 1974) and used in an earlier phase of the current project for the determina- of these materials at levels below 1.0 ppm. However, because of the thermal instabilities and low vapor pressures of RDX and HMX, such a methodology is not satisfactory for the quantitation of these munitions at comparably low levels. Several reports (Doali and Juhasy, 1974; Williams, 1974; Selig, 1973) have appeared in the literature concerning the use of high performance liquid chromatography (HPLC) as a means to avoid the problems of thermal instability and low volatility encountered in the direct GC analysis of these materials, methods which potentially could achieve the high sensitivity necessary for the separation and determination of RDX and HMX in trace amounts. A methodology utilizing HPLC techniques was developed as a part of the work presented in this report and used for the quantitation of HMX, RDX, and TNT in the water samples received during this study. This method has proven to be quite satisfactory in that it does not require sample extraction or derivatization and yet is sensitive to concentrations as low as 0.05 ppm of these munitions, with an analysis time of about 15 minutes.

All analyses were conducted using a Varian 8500 High Pressure Liquid Chromatograph equipped with a DuPont 837 variable multi-wavelength UV detector, and an Infotronics Model CRS-204 digital integrator. The detection wavelength was set at 230 mμ. A 25-cm x 4.6-cm x 0.63 cm Partisil 10-ODS column was used, with a 40 percent methanol/water mobil phase and a 5 ml/hour flow rate. All solvents used in this study were distilled-in-glass analytical grade obtained from Burdick and Jackson Laboratories, Muskegon, Michigan.

As HMX, RDX, and TNT all readily undergo photolytic as well as thermal degradation, all samples were collected in amber bottles and stored at 4 C prior to analysis. Measured aliquots were withdrawn from each sample, filtered through 1.0-μm Millipore Filter Discs (No. FALP 02500), and the residue washed with 1-ml tetrahydrofuran (THF) which was then added to the filtrate. Samples were prepared for LC analysis by simply diluting 100 μl of a 500 ppm solution of 3-nitrophenol (the internal standard) to 1.0 ml with the combined filtrate and wash. Normally, injection volumes of 100 μl were used, with each sample being prepared and run in duplicate. However, for the determination of very low munitions levels (less than 0.300 ppm), 175 μl of these solutions were injected. In all cases, the integrated area ratios of the munition versus internal standard were used to determine concentrations.

The chemical oxygen demand (COD) was determined for these water samples using the procedure outlined in "Standard Methods for Examination of Waste Water", 13th edition (1971). These samples were collected in polyethylene bottles containing 0.5 g $HgCl_2$ added as a preservative, and stored at 4 C prior to analysis.

Standard solutions of each munition were prepared in methanol using authentic samples obtained earlier in this project from Dr. B. E. Hackley, Edgewood Arsenal, Aberdeen Proving Ground, Maryland. The use of a digital integrator coupled to the HPLC detector permitted the accurate determination of the total area of the peaks appearing in the HPLC chromatograms of these materials. Peak "area ratios" were determined by dividing the integrated area obtained by the peak corresponding to each munition by the area obtained for the internal standard. A linear regression analysis of the relationship between the amount of a munition injected and the resultant "area ratio" generated the equations listed below for each munition:

μg TNT injected = 2.4889 (area ratio) + 0.001
correlation coefficient = 1.000

μg RDX injected = 5.3390 (area ratio) + (-0.002)
correlation coefficient = 0.999

μg HMX injected = 4.6760 (area ratio) + (-0.0445)
correlation coefficient = 0.992

In order to calculate the concentrations of HMX, RDX, and TNT in the HAAP water samples received for analysis, a known volume of each sample was injected, the peak area ratios determined for each munition present, and the concentrations of these materials determined using the above equations.

Using a variable wavelength detector, the possibility of increasing detection sensitivity by choosing a detection wavelength to maximize the absorbance for the materials of interest was examined. Spectrophotometric data for ethanolic solutions of several munitions (Schroeder et al., 1951) are given in Table 3.

TABLE 3. SPECTROPHOTOMETRIC DATA FOR SEVERAL MUNITIONS IN ETHANOLIC SOLUTION

Compound	λ (Max) - (nm)	Extinction coefficient	$\times 10^{-3}$
HMX	228-229	21.0	
RDX	213	11.0	
TAX	231-234	6.5	
SEX	227	15.8	
TNT	227	19.7	
2,4-DNT	239-242	14.3	
BSX	227	16.2	
ATX	224-225	16.0	
PETN	225	25.0	

The common detection wavelength for UV detectors in HPLC analysis is the Hg emission line at 254 nm. However, as indicated by the above data, the detector response to TNT, HMX, and RDX will be maximized by using a detection wavelength which more closely approximates the wavelength of maximum absorption for these materials. As the absorption curves of TNT and HMX maximize at about 230 nm, this was chosen as the detection wavelength for use in this study. By similarly choosing a wavelength which maximizes detection sensitivity, other munitions such as those listed in Table 3 may be quantitated at trace concentrations using the general methodology developed in this study.

A lower limit of detection of 0.05 ppm was established using prepared solutions of each munition in methanol. However, the occurrence of interfering materials with peaks in the LC chromatogram appearing very near those of HMX and RDX prevented quantitation of these munitions below 0.10 ppm. Subsequent examination using a 20 percent methanol/water mobile phase was successful in removing the interference problems, but the consequent loss in sensitivity resulting from peak broadening prevented precise and reproducible measurements of peak area. The estimated precision of each analysis is better than 10 percent at concentrations above about 0.10 ppm, but rapidly decreases to no better than 50 percent below this level. Therefore, HMX and RDX concentrations are not reported below 0.10 ppm in these samples. In several cases, the absence of any munition peak whatsoever indicated a concentration level below 0.05 ppm.

Mutagenic Screening. The Ames bacterial mutagenicity test is a bioassay designed to detect potential mutagens by means of a special set of five Salmonella typhimurium strains developed by Dr. Bruce Ames. Specifically, these are TA-1535, TA-1537, TA-1538, TA-93, and TA-100. The assay is based on the property of these five strains for reversion from a histidine requiring state to prototrophy due to exposure to various classes of mutagens. The histidine deficient varient strains are used to detect frame shift reverse mutations (TA-1537, -1538, and -98) or base pair substitutions (TA-1535 and -100). These tester strains were developed for their sensitivity and specificity to be reverted back to the wild type by particular mutagens.

The assay has been adapted for use in detecting compounds which may be potential mutagens. It has recently been documented that most compounds that act as carcinogens in mammals also act as mutagens in bacterial systems. A significant percentage of known carcinogenic compounds are not active carcinogens in the parent form but require enzymatic alteration to an active moiety. Mammalian microsomal hydroxylase systems are responsible for this activation. Since these specific bacteria do not have the mammalian microsomal enzyme system, mammalian liver homogenates are added to the system to activate the non-mutagenic parental compounds to possible mutagens.

The activation system for mutagenesis screening consisted of Arochlor 1254 induced microsomes derived from rat livers. Induction was accomplished by a single intraperitoneal injection of Arochlor (diluted 200 mg/ml of corn oil) into individual rats 5 days before sacrifice at a dosage of 0.5 mg/g of body weight. The rats were deprived of food and water 24 hours before sacrifice. The rats were then stunned by a blow on the head and decapitated.

The livers were removed aseptically from the rats and placed into a cold preweighed beaker containing 10 ml of 0.15M KCl. The livers were swirled in this beaker and then removed with forceps to a second beaker containing 3 ml of the KCl solution per gram of wet liver weight. The livers were then minced with sterile scissors, transferred to a chilled glass homogenizing tube and homogenized by passing a low speed motor driven pestle through the livers a maximum of three times. The homogenates were than placed in cold centrifuge tubes and centrifuged for 10 minutes at 9000 G at 4 C. The resulting supernatant was decanted, aliquoted in 3-ml amounts to small culture tubes, quickly frozen in dry ice, and stored -80 degrees in a Revco freezer. Sufficient microsomes for use each day were thawed at room temperature and kept on ice before and during use.

The liver microsomes were incorporated into a mix which was prepared according to the recommendations of Ames. The microsomal mix contained per ml: liver microsome preparation S-9 (0.15 ml), $MgCl_2$ (8 μ moles), KCl (33 μ moles), glucose 6 phosphate (5 μ moles), NADP (4 μ moles), and sodium phosphate pH 7.4 (100 μ moles). Stock solutions of NADP (0.1M) and glucose-6-phosphate were prepared with sterile water, aliquoted in appropriate amounts, and maintained in a Revco freezer. The stock salt solutions were prepared, autoclaved, and refrigerated. The S-9 mix was prepared fresh each day and was maintained on ice before and during use.

The Salmonella typhimurium mutant strains were obtained from Dr. B. Ames. Upon receipt, broth stock cultures of each strain were grown, aliquoted in small vials and stored in a Revco freezer. At the beginning of these investigations new bacterial cultures were obtained from this stock supply. Subsequent to confirmation of biochemical

activity and spontaneous reversion rates, master cultures of each of the strains were prepared and used on the origin of weekly preparations of working cultures. All broth cultures were nutrient broth (Difco) supplemented with 0.5 percent NaCl. The bacterial cultures to be used for an assay were prepared by inoculating 0.1 ml of each tester strain into 100 ml of nutrient broth and incubating the culture in a water bath shaker for 16 to 20 hours.

The selective basal medium for histidine requiring strains used in mutagenesis assays was a 1.5 percent Bacto-Difco agar in Vogel-Bonner Medium E with 2 percent glucose.

The top agar (0.6 percent Difco agar, 0.5 percent NaCl) was prepared in 10 ml aliquotes, autoclaved, and stored at room temperature. Before use in mutagenesis assays the agar was melted and 10 ml of a sterile solution of 0.5 mM l-Histidine-HCl and 0.5 mM biotin was added to the molten top agar and mixed thoroughly.

A number of control tests were conducted to ascertain the efficiency of the test and the sterility of the test components. Positive control dose response assays were performed with each of the tester strains with the appropriate positive control chemical. The purpose of the positive control assay was to check the performance of the test components both with and without microsomal activation and to provide a standard against which any activity of the test water sample may be compared.

Immediately upon receipt, the water samples were refrigerated in the dark. Each of the samples was filter sterilized before being assayed because of large amounts of extraneous sedimentary material in the sample. The mutagenicity determinations were made in triplicate for each water sample evaluated. The assays were conducted with each of the five tester strains both with and without the presence of the microsomal activation system. Sterility control checks and positive control assays were included.

The assay was conducted in the following manner. A 0.1 ml aliquot of the broth culture of the tester organism was added to 2 ml of molten top agar which had been supplemented with a trace of histidine

and biotin. In the non-activation tests, 50 µl of the water sample was added to the molten top agar and subsequently poured over a minimal medium agar plate. In activation mutagenesis assays, dose response curves were prepared for each tester strain in the presence of 100, 50, 20, 10, and 5 µl of test water. In these tests 0.4 ml of the microsomal mixture was added to the top agar after the tester strain and the selected concentration of the water sample had been added and just before pouring.

The poured top agar was permitted to solidify before the plates were incubated. Following an incubation period of approximately 72 hours, the number of colonies growing on each plate was counted.

Bioassay Test. Onsite aquatic bioassays were conducted at HAAP in a mobile facility housed in a 30-foot aluminum trailer. The unit was insulated and had air-conditioning to control inside temperatures. Shelving was positioned along one wall of the trailer to provide maximum bench area for bioassay test vessels. The trailer was equipped with two 100-gallon fiberglass holding tanks for the acclimation of test fish. Additional counter space was available for sample preparation and equipment storage.

Bioassay tests were 96-hour LC_{50} static acute tests of appropriate waste stream influents to and effluents from the pilot treatment plant. Methodologies utilized adhered to the extent practicable to those described and recommended by the U.S. Environmental Protection Agency (1975) and the American Society of Testing Materials (Draft Report). Fathead minnows (Pimephales promelas) were purchased from Mal-Bro hatcheries located in Blountville, Tennessee and Anderson's Minnow Farm in Lonoak, Arkansas.

Test concentrations for the various wastewaters were determined based on results obtained from 24-hour range finding tests. The 96-hour tests included five wastewater concentrations and one control. Exceptions were made in 3A and 6A tests when sufficient test water was not available at the beginning of the test. South Fork Holston River water was used as the dilution media. Screening tests using 40 fathead minnows showed no mortality after 120 hours in Holston River water. The river water was also used as the control water for each test set. Each test was run in duplicate.

Test concentrations were mixed at the trailer site using volumetric techniques. Eighteen liters of solution were used in each 5-gallon bottle. Initially aeration was avoided in the test chambers. However, after several tests in which the dissolved oxygen (D.O.) concentration dropped below the predetermined lower limit of 4.0 mg/l, aeration was begun to maintain sufficient D.O. levels. Tests conducted without aeration utilized five (5) minnows in each chamber. When it was decided to use artificial aeration in the form of aquarium aerator pumps, ten (10) minnows were used.

Prior to all tests, test organisms had been acclimated to the dilution water and trailer temperature fluctuations (19°C - 23°C, generally) for 4 or more days. The same temperature range was maintained in the 5-gallon chambers during the actual tests. Acclimation and testing was done under artificial lighting, illuminated 8 hours of every day.

Fish were fed commercial aquarium food during acclimation. Specimens were not fed for 48 hours prior to utilization in a bioassay test.

Disease problems (fin rot) encountered in the holding tanks were treated by adding commercially obtained furacin to the holding tanks. The incidence of fin rot decreased dramatically after the initiation of the furacin treatments.

Test organisms were added to test chambers in the order established by a random numbers table. Each series of test chambers was also placed in random order prior to the addition of the fish. Initial temperature, dissolved oxygen and conductivity readings were taken and recorded on test data sheets. Mortality, temperature and D.O. were recorded at 24-hour intervals up to 96-hours or when a mortality threshold was reached. Dead specimens were removed when observed. Criteria defining death was cessation of all movement and lack of response to gentle prodding.

Probit Analysis

Probit analysis, well suited for use in bioassay studies (Sokal and Rohlf, 1969), was used to fit a line to the percentage of mortalities accumulated by the last day of each run in response to the different effluent

concentrations. The purpose of the probit analysis was also to derive the LC_{50} value for each effluent, that is, the concentration of the effluent which would produce exactly 50 percent mortality. A computer program (IBM, 1970) was used to calculate the values of a and b in the equation $Y = a + bX$. X represents the effluent concentration and Y is the normal deviate of the proportion of fish dying at the concentration, with the value of 5.0 added (to eliminate the possibility of Y being negative). After a and b are calculated, the LC_{50} value can be easily derived. The proportion of 50 percent mortality corresponds to a normal deviate of 0 and, therefore, a Y value of 5, so LC_{50} is simply the X for which $a + bX = 5$. That is, $LC_{50} = (5-a)/b$. A 95 percent confidence interval was also computed for each LC_{50} value (Bliss, 1952).

Each probit analyses was run twice, once with the X values untransformed and once with the X values transformed by logarithms. The chi-square "goodness-of-fit" test for the agreement of the predicted line with the actual data showed that the untransformed data resulted in a better fit. However, in some analyses, the predicted responses differed significantly from the actual responses at the 95 percent level, regardless of whether or not the concentrations were log-transformed. Such cases were noted, and the coefficients of the equations were always reported for the lines fitted to the untransformed concentrations.

Separate probit analyses were run on the data from each of the replicate runs. In addition, probit analyses were performed on the data obtained by pooling each pair of replicates in order to smooth out irregular variations due to random differences between the replicates. The pooling was done by summing the mortalities at each concentration over the two replicates, and by computing the proportion of deaths using twice the number of fish per tank.

The upper and lower limits of the 95 percent confidence interval for each LC_{50} value were calculated after using a t-statistic to determine whether or not the slope of the probit equation line was significantly different from zero. If the slope was not significant, a true 95 percent confidence interval could not be calculated. A special note was made of the probit analyses which produced such a result, and a substitute "confidence interval' was determined as follows: the lower limit was taken

to be the highest effluent concentration at which no mortality occurred, and the upper limit was taken to be the lowest effluent concentration in which 100 percent mortality occurred.

Also, for some bioassay tests all the responses at the lower concentrations were zero mortality, and all the responses at the next higher concentrations were 100 percent mortality. In such an "all-or-nothing" situation, no actual probit analysis could be performed, so there was no regression line from which an LC_{50} value could be calculated. In these cases the LC_{50} was taken to be the average of the two consecutive concentrations between which there was a jump from zero to 100 percent mortality. These two concentrations were also reported as the lower and upper limits of a "substitute confidence interval", reported in place of a 95 percent confidence interval for the LC_{50} value.

A number of probit analyses produced 95 percent confidence intervals for which the limits were less than zero or greater than 100. When this occurred, the actual calculated values for the limits were not reported, since concentrations outside the range of zero to 100 percent are not meaningful.

Munitions Constituent Comparisons

Battelle's Columbus Laboratories and HAAP data for HMX, RDX, TNT and COD were compared. For each of these parameters, a paired T-test using a two-tailed test of significance was performed to determine whether the data from the two laboratories varied in a consistent way. The HMX values from Battelle proved to be higher than those from Holston by an average of 0.6 ppm, which had a significance level of 99.5 percent. No other parameters showed significant differences, and Battelle's data were used in subsequent statistical analyses using these parameters.

Correlation Coefficient.

Pearson's "r" correlation coefficients and their associated significance levels were computed on all possible paired combinations of munitions and water quality parameters (excluding TNT since it was not

detected in any of the samples). All munitions and water quality parameters except pH showed a considerable amount of positive skewness, so all data except pH were transformed by taking $\log_{10}$ (X+1) before computing the correlation coefficients. This transformation succeeded in bringing the skewness coefficients for all parameters closer to zero. This step was taken to prevent the occurrence of misleadingly high correlation coefficients which can result from a very small number of outlying points.

Pearson's "r" coefficients were also computed to correlate each munition and water quality parameter with the LC_{50} values resulting from the probit analyses which were performed on pooled replicates of bioassay tests. To prepare the data for these correlations, the LC_{50} value from each bioassay was matched with the water chemistry analysis on the effluent used in that particular bioassay. (LC_{50} values that were well over 100 percent were excluded from the calculations.) All the LC_{50} values were then transformed by logarithms in the same manner as were the water quality parameters. Again, this was done to reduce skewness since the actual, calculated LC_{50} values ranged over two orders of magnitude.

RESULTS AND DISCUSSION

Test Water Analysis

Battelle's Munitions Constituent and COD Analysis

All of the water samples examined in this study contained less than 6.0 ppm of all the munitions selected for determination. In fact, TNT never occurred above the detection limit of 0.05 ppm in any of the samples examined. From the limited number of samples examined, Area A and A+B wastes appear to be characterized by generally higher levels (~0.1 to ~5.0 ppm) of HMX and RDX as compared with the treated 3A and 6A wastes. The treatment wastes generally ranged from < 0.05 to 0.7 ppm with the notable exceptions of HMX concentrations in 6A samples

on June 12 and June 22, and in 3A samples on June 15 and June 23 (see Table 4). Munitions waste concentrations were too low to be useful in any estimate of removal efficiency.

A sample of the 6A effluent (June 25) was spiked in the field with ~ 10 ppm RDX prior to the bioassay test. Subsequent HPLC analysis of samples of this test solution and one taken at completion of the bioassay gave results which were considerably lower than 10 ppm (see Table 4). The inconsistency between the spiked RDX concentration and that determined by HPLC analysis may be a result of the known thermal and photolytic degradation of this munition. This degradation process is perhaps evidenced by the increase in the concentration of a suspected degradation product in the spiked sample as observed when the chromatogram of the spiked and corresponding unspiked samples are compared in Figures 2a and 2b. For reference purposes Figure 2c presents a chromatogram of Holston River water as a control and Figure 2d a chromatogram of a standard mixture of the known munitions to illustrate each compound's retention time. The occurrence of higher HMX concentrations in the samples spiked with RDX than in the unspiked samples was observed (see Table 4) and may be a result of contamination of the RDX added to the spiked sample with traces of HMX. COD values varied over a wide range, being generally an order of magnitude higher in the Area B and A+B samples than the ~10-100 ppm range observed in the waste treatment effluents (3A and 6A). Values of several thousand ppm were determined in some Area A waste samples.

The authenticity of the peaks appearing at the retention time corresponding to that of known munitions may be verified by collecting the LC fraction and analyzing this fraction by mass spectrometry. This verification was done for the HMX peaks appearing in the chromatograms of Area A waste samples. The LC fractions corresponding to the HMX peak were collected, extracted with ethyl acetate, and analyzed by direct probe chemical ionization mass spectrometry (CI-MS) using isobutane as the reagent gas. The results of this analysis were compared with those obtained with authentic samples of HMX in ethyl acetate and were found to be identical. However, neither sample duplicated the fragmentation patterns obtained under similar conditions with pure, solid HMX, and reported in the literature (Yinon, 1974).

TABLE 4. CHEMICAL ANALYSIS OF HAAP WATER SAMPLES[a]

	HMX (ppm)	RDX (ppm)	TNT (ppm)	COD (ppm)
Area A				
June 2, 1976	3.61	0.72	<0.05[b]	4490
June 7, 1976	1.11	0.43	"	4400
June 11, 1976	5.40	0.82	"	3790
June 15, 1976	4.92	1.90	"	4090
	0.31	1.63	"	600
June 16, 1976[d]	0.85	<0.05	"	170
June 19, 1976	2.92	0.52	"	3440
June 19, 1976[d]	0.23	<0.05	"	170
Control-Holston River Water	<0.05	<0.05	<0.05	58.0
Area 6A				
June 3, 1976	<0.1	<0.05	<0.05	116
June 4, 1976	0.2	<0.05	"	192
June 10, 1976	<0.1	<0.05	"	58.0
June 12, 1976	2.10	"		68.0
June 19, 1976		Not done		39.0
June 22, 1976	1.56	<0.05	<0.05	35.0
June 25, 1976	0.20	4.75	"	50.0
Area B				
June 2, 1976	<0.1[c]	<0.1	<0.05	469
June 7, 1976	1.85	1.34	"	176
June 17, 1976	0.72	<0.05	"	156
June 23, 1976	0.42	1.70	"	433
Area A+B				
June 2, 1976	0.48	<0.1	<0.05	644
June 7, 1976	2.46	1.64	"	
June 7, 1976	2.46	1.59	"	808
June 14, 1976	2.13	<0.1		730
June 14-16, 1976	0.14	<0.1		100
Area 3A				
June 3, 1976	0.42	<0.05	<0.05	112
June 4, 1976	0.25	<0.1	"	112
June 9, 1976	0.72	0.1	"	60.0
June 11, 1976	0.24	0.1	"	76.0
June 15, 1976	1.22	<0.05	<0.05	66.0
June 15-16, 1976	0.43	<0.05		58.0

TABLE 4. (Continued)

	HMX (ppm)	RDX (ppm)	TNT (ppm)	COD (ppm)
Area 6A (Cont.)				
June 25, 1976	<0.1	<0.05	"	29.0
June 29, 1976	0.46	5.17	"	42.0
June 29, 1976	<0.1	<0.51	"	40.0
Area 3A (Cont.)				
June 20, 1976	0.64	<0.05		430
June 23, 1976	2.07	<0.05		41.0

(a) The concentration of the munition is below the lower limit of detection of 0.05 ppm, as no peak was observed in the LC chromatogram.

(b) The lack of reproducibility experienced at low levels prevented quantitation below 0.10 ppm.

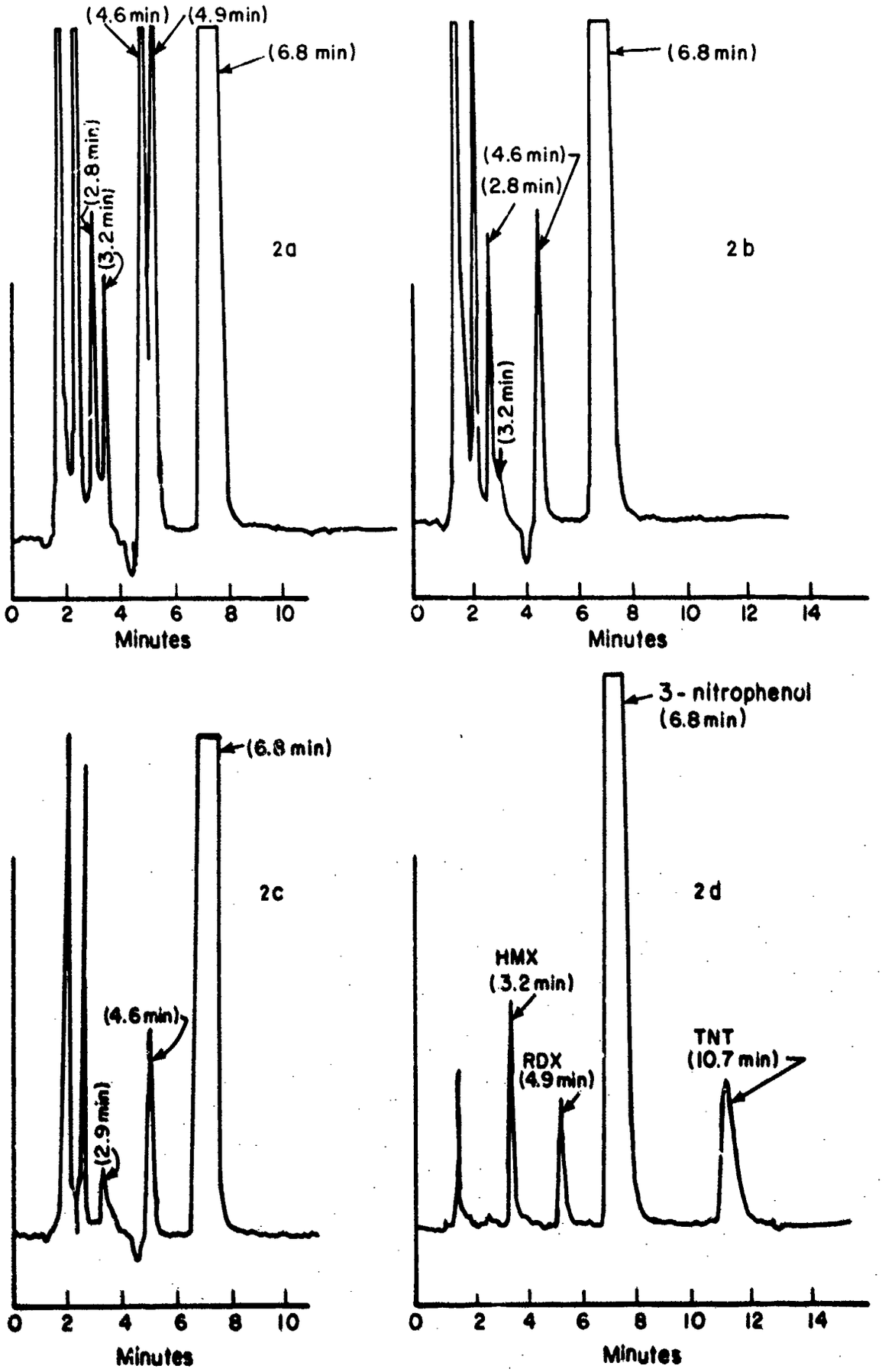


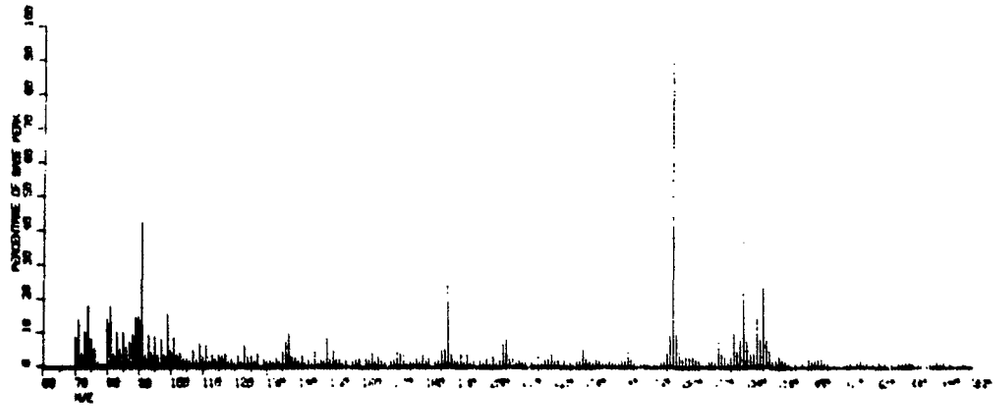
FIGURE 2. HPLC CHROMATOGRAMS OF HAAP WATER SAMPLES (2a) AREA 6A, 6/29/76 ①, (2b) AREA 6A, 6/29/76 ②, AND (2c) CONTROL-HOLSTON RIVER WATER, AND (2d) A MIXTURE CONTAINING 1 PPM HMX, RDX, TNT, AND 50 PPM 3-NITROPHENOL (INTERNAL STANDARD)

when we examined crystalline HMX (which had not been dissolved in ethyl acetate) the mass spectral fragmentation patterns produced were in agreement with the literature data (see Figure 3). Further work is required to clarify these results (e.g., spectral matching using electron impact mass spectrometry combined with laboratory studies concerning the reactivity of HMX under controlled conditions). In any case it appears that the same material is present in both the HPLC fraction and the solution of known HMX, and the accuracy of the LC identification is verified.

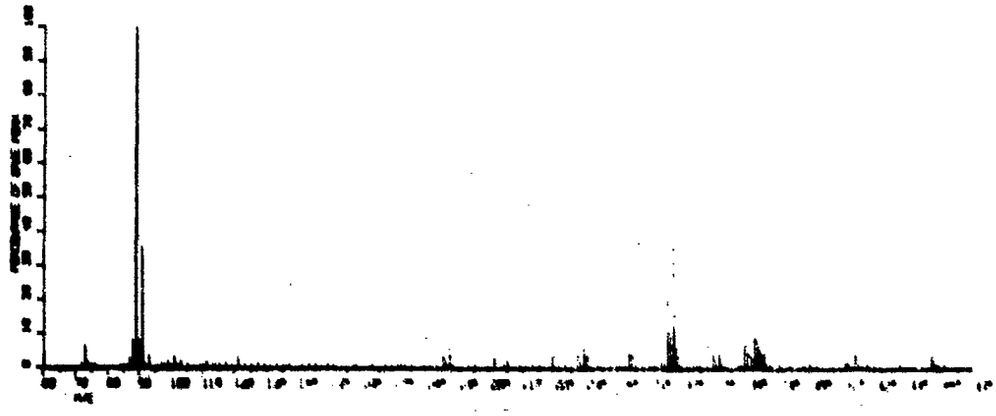
Additional varification of the authenticity of the HMX and RDX peaks was accomplished during an examination of these fractions collected from 6A, effluent June 29, 1976 (see Appendix A). A CI-MS analysis of the ethyl acetate extract of these LC fractions was performed using CH_4-NH_3 as the reagent gas. In this examination matching spectral patterns were obtained for the HMX fraction and known HMX, and for the RDX fraction and known RDX. In addition, these patterns matched those reported by Yinon, (1974), although obtained under somewhat different conditions. The HMX fraction in 6A, June 29, corresponds in retention time to the peak identified as HMX in the Area A samples.

During the initial analysis of the HAAP samples, conducted during August, 1976, several materials other than the munitions were consistently observed in the water samples submitted for analysis, and the levels of two of these materials appeared to coincide with the levels of intact munitions observed. It was therefore considered important to characterize these materials.

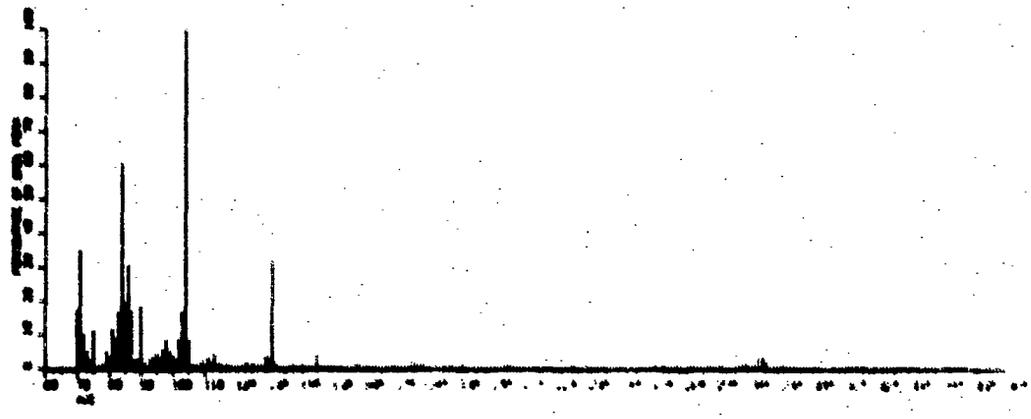
The two materials of concern are illustrated in the chromatograms presented in Appendix A, Figure A-1a, by the peaks with retention times of 2.8 and 4.6 min, which appear very close to the peaks corresponding to HMX and RDX, respectively. Upon examining the chromatograms of several water samples containing varying concentrations of HMX and RDX, the levels of these two unidentified constituents appeared to rise and fall with the amount of HMX and RDX as illustrated in Figures A-1a and A-1b, indicating a relationship between the presence of the intact munition and these unknown materials. However, in October, 1976, (some 3.5 months later) the same water sample described above was re-examined by HPLC, and a



3a



3b



3c

FIGURE 3. CHEMICAL IONIZATION MASS SPECTRA (USING ISOBUTANE AS REAGENT GAS) OF (3a) HMX IN LC FRACTION OF AREA A, 6/2/76, (3b) AUTHENTIC HMX REMOVED FROM ETHYL ACETATE SOLUTION, AND (3c) CRYSTALLINE HMX

different chromatogram was obtained. As shown in comparing Figures A-1a and A-1c, the peak at 4.6 min was no longer observed and the relative intensities of several of the remaining peaks had changed substantially. However, no new peaks were observed in these latest chromatograms and the concentrations of HMX and RDX remained essentially unchanged. Several of these water samples were examined, all giving the same result. Although the water samples were stored at a constant 4°C in amber glass bottles and optimized analytical procedures were used duplicating those employed in the previous analysis, identical chromatograms were not achieved after a period of 3.5 months. A more careful examination using a modified HPLC procedure revealed the presence of a very small peak which eluted just prior to RDX and which appeared to be the remains of the peak occurring at 4.9 min in Figure A-1a.

Although these samples had obviously undergone some significant change with time, an attempt was made to characterize what appeared to remain of the materials of interest. The 6A water sample from June 29, 1976 was chosen as the sample for examination because of its initially observed high levels of HMX, RDX, and the two constituents with retention times of 2.8 and 4.9 min (see Figure A-1c). A chromatogram of the ethyl acetate extract of this sample is shown in Figure A-1e. The A, B, C, and D fractions which were collected correspond to the aforementioned materials, respectively.

Mass spectral (MS) and nuclear magnetic resonance (NMR) analysis of these fractions were conducted and the results are shown in Figures A-3 and A-4 in the Appendix. Only Fractions B, C, and D contained sufficient material to permit these analyses. The MS analysis of Fractions C and D gave identical results, implying that Fraction C overlapped with the highly concentrated RDX Fraction D and therefore contained primarily RDX also. However, in each case anomalous peaks were observed in the range m/e 270 to 300 which were not observed with crystalline RDX (Figure A-3e). These peaks may be due to some contaminant arising from the isolation procedure. However, when examined by NMR, Fraction C is seen to contain primarily a material which is not RDX. Both crystalline RDX and Fraction C contain a CCl_4-soluble species which gives a spectrum very different from

that of RDX. This material may be some structurally unsymmetrical isomeric form of RDX present as a degradation product or a by-product of RDX manufacture. Such a material might yield an identical mass spectral fragmentation pattern as RDX, but the NMR spectrum would be substantially different from the singlet observed for RDX if this material does not possess the same high degree of symmetry as RDX. Substantiating evidence in this regard might be obtained by similarly examining the NMR spectrum of Fraction D for the presence of these multiplets. However, because there is some question as to the integrity of these water samples, a rigorous characterization and interpretation of these collected fractions does not seem warranted. The primary thermal and photolytic decomposition products of HMX and RDX are H_2O and CH_2O which would not be detected by the HPLC method employed in this study. Such a decomposition scheme might explain the apparent change observed in these water samples with time, if the constituents of interest are but a part of a series of intermediates in the HMX and RDX degradation process. Hence, the materials collected and examined in the present study may not be the same as those originally observed in these samples.

Few conclusions can be drawn at this time as to the nature of these constituents of the HAAP water sample. Fresh samples should be collected and the nature and levels of the unidentified constituents examined as a function of munitions concentration and time. By combining this study with a controlled study of the thermal, biological, and photolytic degradation of HMX and RDX and an assessment of likely by-products in the manufacturing process, a more complete picture of the environmental fate of these munitions and their manufacturing waste stream may be obtained.

Bioassay Effluent Water Quality

Water quality parameters were measured on effluent test water by HAAP chemists in the plant laboratory. Table 5 presents the results of these analyses and the BCL munitions constituent and COD data. Waste

TABLE 5. HAAP BIOASSAY TEST WATER QUALITY AND MUNITIONS CONSTITUENTS WATER QUALITY PARAMETERS (ppm)

Effluent	Test No.	Test Date	RCL Analysis				HAAP Analysis											
			HMX	RDX	TNT	COD	BOD (filtered)	BOD	pH	NH_3-N	TKN	D.O.	PO_4	NO_3	NO_2	SS	Settle Solids	Total Solids
Area A																		
	1	6- 2-76	3.61	0.72	<0.05	4,440	—	2,644	3.4	123.2	153.4	0	—	35.2	65.0	—	0.9	1,398
	2	6- 7-76	1.11	0.43	<0.05	4,440	3,130	3,175	3.8	117.0	148.8	0	426	43.0	—	—	23.0	2,732
	3	6-11-76	5.40	0.82	<0.05	3,790	—	3,264	3.6	113.3	130.2	0	435	40.5	8.0	—	0.1	1,742
	4	6-15-76	4.92	1.90	<0.05	4,090	—	2,684	3.6	100.8	133.2	0	400	37.0	47.0	—	15.0	3,358
	5	6-19-76	2.92	0.52	<0.05	3,440	—	2,785	6.9	57.1	68.4	—	—	—	—	—	0.8	816
Area B																		
	6	6- 2-76	<0.1	<0.1	<0.05	469	259	266	6.8	34.7	62.6	0.6	—	3.0	1.9	132	0.7	398
	7	6- 7-76	1.85	1.34	<0.05	176	136	96	6.8	2.9	6.0	0.6	33.0	6.6	—	164	2.0	378
	8	6-17-76	0.72	<0.05	<0.05	156	141	148	7.5	9.6	13.0	—	—	—	0	—	0	266
	9	6-23-76	0.42	1.70	<0.05	433	104	255	8.0	5.9	9.2	0	14.0	122	—	142	5.0	660
A + B																		
	10	6- 2-76	0.48	<0.1	<0.05	644	323	298	6.5	27.3	74.8	4.8	—	7.1	9.9	—	3.5	826
	11	6- 7-76	2.46	1.59	<0.05	838	—	—	6.2	27.3	35.4	4.8	53.4	12.7	—	—	4.5	712
	12	6-14-76	2.13	<0.1	<0.05	730	236	258	6.8	15.6	24.0	3.5	50.0	9.0	17.0	—	5.3	1,020
1A																		
	13	6- 4-76	0.25	<0.1	<0.05	112	4	12	7.5	7.3	19.2	—	15.9	—	—	—	—	480
	14	6- 9-76	0.72	0.1	<0.05	60	18	39	7.7	6.0	10.3	—	30.9	—	—	1,284	—	—
	15	6-12-76	0.24	0.1	<0.05	76	32	49	7.7	3.4	7.0	—	48.8	22.0	10.5	—	—	422
	16	6-15-76	1.22	<0.05	<0.05	66	10.5	42	7.2	0	3.6	3.7	52.8	86.0	8.8	—	—	834
	17	6-20-76	0.64	<0.05	<0.05	430	5	6	7.6	0	2.5	—	28.8	98.0	5.8	—	—	442
6A																		
	18	6- 3-76	<0.1	<0.05	<0.05	116	262	326	7.5	15.1	31.1	0.4	20.6	11.8	1.5	—	—	590
	19	6-10-76	<0.1	<0.05	<0.05	58	10.5	12	7.6	8.4	12.5	0.2	27.0	8.7	2.8	0.23	—	586
	20	6-12-76	2.10	<0.05	<0.05	68	8	8	7.5	9.0	14.0	—	48.6	16.0	3.8	—	—	400
	21	6-19-76	—	—	—	39	14	17	7.5	5.0	7.6	—	—	35.0	4.2	—	—	502
	22	6-22-76	1.56	<0.05	<0.05	35	8	15	7.8	0	1.3	0.4	27.6	33.5	8.0	—	—	616
	23	6-25-76	<0.1	<0.05	<0.05	29	4	4	8.8	0	1.9	—	—	30.0	<1.0	—	—	502
	24	6-25-76(a)	0.46	5.17	<0.05	50	5	10	8.6	0	2.9	—	—	32.0	0	—	—	—

(a) Spiked with 10 ppm RDX

waters from both Area A and Area B were high in COD, BOD, NH_3-H, TKN, PO_4, nitrates, nitrites, and solids. The Area A wastewater was also low in pH and D.O. with values being approximately 3.5 and 0.0 respectively. Area B effluent water had pH values near 7.0 and quite low D.O. values.

Values for HMX and RDX in the wastewaters were consistently higher in the Area A wastes than in the Area B. Test water concentrations of HMX in Area A wastes ranged from 1.11 to 5.40 while Area B water had lower values ranging from <0.1 to 1.85. RDX values, while more similar in concentration between the two area effluents than were HMX levels, were still usually higher in the Area A waste. This was a reversal of what was expected as Area B was the explosive manufacturing area.

Biologically treated A+B wastewater (3A and 6A effluents) had generally lower values of munitions constituents, COD, BOD, ammonia, nitrite, phosphate, and TKN. pH values in effluents 3A and 6A ranged between 7.2 and 8.8. Dissolved oxygen values were still usually quite low in both treatment plant effluents. Total solids levels in the 3A and 6A effluents were similar to the combined A+B wastewater.

Water Quality Correlations

Table 6 displays the internal correlation coefficients among munitions and water quality parameters. (The 1.00's along the diagonal of the matrix are displayed for visual balance in the table, even though these values are redundant because every variable has a correlation of 1.00 with itself.) The results reveal a subset of ten variables which are all strongly correlated with one another. These variables are HMX, COD, BOD (filtered), BOD, pH, NH_3, total Kjeldahl N, PO_4, NO_2, and total solids. These variables are all positively correlated with each other (except pH). On the other hand, pH was negatively correlated with all other variables, since high values of pH tended to be associated with low values of all the other variables, and vice versa.

Suspended solids, settleable solids, and D.O. were not significantly correlated with any other variables. This may have resulted from

TABLE 6. SIGNIFICANT CORRELATION COEFFICIENTS AMONG MUNITIONS AND WATER QUALITY PARAMETERS[a]

	MNX	RDX	COD	BOD Filtered	BOD	pH	NH_3	Total Kjeldahl N	DO	PO_4	NO_3	NO_2	Suspended Solids	Settleable Solids	Total Solids
MNX	1.00														
RDX		1.00													
COD	.65		1.00												
BOD - Filtered			.89	1.00											
BOD	.57		.95	.98	1.00										
pH	-.71		-.85	-.77	-.81	1.00									
NH_3	.49		.90	.80	.87	-.81	1.00								
Total Kjeldahl N	(.46)		.90	.80	.88	-.83	.98	1.00							
DO									1.00						
PO_4	.71		.80	(.58)	.75	-.95	.73	.75		1.00					
NO_3											1.00				
NO_2	.69		.67		.57	-.75	(.51)	(.52)		(.56)		1.00			
Suspended Solids													1.00		
Settleable Solids														1.00	
Total Solids	.60	(.47)	.75	(.50)	.70	-.84	.63	.67		.86		.76		(.60)	1.00

(a) Values in parentheses are significant at the 95 percent level; all other values are significant at the 99 percent level or greater. Blanks indicate that the coefficients were not significant at the 95 percent level.

the fact that more data were missing for each of these three variables than for any other parameters. RDX was not significantly correlated with any other parameter except total solids.

Bioassay Tests

LC_{50} Values

Table 7 presents the results of probit analyses performed on the bioassay test data. As expected, the LC_{50} value for each set of pooled replicates is numerically close to the average of the two LC_{50} values for the separate replicates.

Since low LC_{50} values indicate high levels of toxicity, Table 7 shows that the composited effluent from Area A is the most toxic of the effluents at HAAP. All the runs using Area A wate· resulted in LC_{50} values on the order of 1 percent concentration, with a fairly small amount of sample variation. The effluent from Area B was considerably less toxic but its LC_{50} values showed a wide degree of sample variation ranging from 6.2 percent to 43.8 percent. (One LC_{50} value for Area B on June 17 was calculated to be 79.4 percent; this was much higher than 32 percent, the highest concentration actually tested, so it cannot be considered valid.) The combined Area A and Area B (A+B) wastewater was intermediate in toxicity between the separate Area A and Area B, with LC_{50} values ranging from 1.43 percent to 14.0 percent.

Virtually no toxic effects were observed in about half of the bioassays using treatment plant effluents 3A and 6A. Toxicity could not be quantified in the form of an LC_{50} value when less than half of the fish died in each of the different concentrations. For some of the bioassays in which this occurred, there was a small increase in the numbers of deaths as the concentration increased, but in many cases there were no deaths even at the 100 percent concentration. The probit analyses performed on such data yielded LC_{50} values greater than 100 percent; in those cases the number of mortalities occurring at 100 percent were reported in the table rather than the LC_{50} value.

TABLE 7. RESULTS OF PROBIT A

Effluent,	Date	Replicate 1 Intercept	Slope	96 Hr LC_{50}	Bounds of 95% Confidence Interval	Footnotes Pertaining to Analysis	Intercept	Slope	96 LC
A	6/2	4.04	1.282	0.75	0.00, 3.20	c	—	—	0.
A	6/7	—	—	0.78	0.56, 1.00	b	—	—	0.
A	6/11	2.59	1.635	1.48	0.56, 3.20	c	1.38	4.146	0.
A	6/15	3.64	1.007	1.35	0.73, 1.98		3.94	1.172	0.
A	6/19	3.75	2.143	0.58	0.29, 0.88	d	3.54	2.583	0.
B	6/2	3.59	0.071	19.9	7.77, 32.0		2.06	0.103	28.
B	6/7	2.51	0.097	25.7	13.5 , 38.0		3.10	0.043	43.
B	6/17	4.23	0.010	>32.0	— —	e	4.69	0.008	(36.
B	6/23	3.54	0.237	6.17	3.28, 9.06		3.84	0.114	10.
A+B	6/2	—	—	14.0	10.0, 18.0	b	1.07	0.324	12.
A+B	6/7	4.16	0.588	1.43	0.00, 10.0	c	3.97	0.376	2.
A+B	6/14	3.72	0.123	10.4	6.33, 14.5		4.15	0.102	8.
3A	6/4	—	—	(none dead at 100%	— —		—	—	(none d at 100
3A	6/9	3.20	0.023	78.7	49.7 , (>100.)		3.08	0.021	92.2
3A	6/12	—	—	(none dead at 100%	— —		—	—	(2/10 d at 100
3A	6/15	3.82	0.015	80.8	9.81, (>100.)	d	4.04	0.026	36.7
3A	6/20	—	—	89.0	78.0 ,100.	b	—	—	(2/10 d at 100
6A	6/3	—	—	(1/5 dead at 100%	— —		3.35	0.018	89.5
6A	6/10	0.34	0.066	70.9	61.2 , 80.6		3.91	0.014	78.8
6A	6/12	1.30	0.089	41.5	32.5 , 50.5		4.62	0.023	16.2
6A	6/19	—	—	(none dead at 100%	— —		—	—	(1/10 d at 100
6A	6/22	—	—	(none dead at 100%	— —		—	—	(none d at 100
6A	6/25	—	—	(none dead at 100%	— —		—	—	(none d at 100
6A+RDX	6/25	2.84	0.030	71.4	51.9 , 90.8		2.41	0.030	64.5

(a) Intercept = a and slope = b in the probit equation Y = a + bX.

(b) Probit analysis could not be performed; all responses were either zero or 100% mortality (see methods).

(c) Slope was not significant so a true confidence interval could not be constructed (see methods).

(d) Goodness-of-fit test rejected the fitted values at the 95% significance level.

(e) The calculated LC_{50} value is greater than 32% (the highest concentration used); slope was not significant.

(f) The calculated LC_{50} value is slightly greater than 100%.

T ANA F PROBIT ANALYSES ON HAAP BIOASSAY DATA[a]

96 Hr LC_{50}	Replicate 2 Slope	96 Hr LC_{50}	Bounds of 95% Confidence Interval	Footnotes Pertaining to Analysis	Replicates 1 & 2 Pooled Intercept	Slope	96 Hr LC_{50}	Bounds of 95% Confidence Interval	Footnotes Pertaining to Analysis
0.55	—	0.55	0.10, 1.00	b	3.55	2.323	0.62	0.31, 0.93	
0.78	—	0.78	0.56, 1.00	b	—	—	0.78	0.56, 1.00	b
0.87	4.146	0.87	0.68, 1.06		1.23	3.770	1.00	0.83, 1.17	
0.90	1.172	0.90	0.33, 1.47		3.82	1.051	1.13	0.74, 1.51	
0.57	2.583	0.57	0.36, 0.78		3.65	2.350	0.58	0.39, 0.76	d
28.6	0.103	28.6	10.0 , 56.0	c	3.18	0.075	24.4	17.3 , 31.5	
43.8	0.043	43.8	23.5 , 64.2		3.07	0.055	34.8	24.3 , 44.3	
(36.7)	0.008	(36.7)	— —	e	4.48	0.009	>32.0	— —	e
10.1	0.114	10.1	5.87, 14.3		3.79	0.145	8.33	5.90, 10.8	
12.1	0.324	12.1	1.00, 18.0	c	0.37	0.360	12.9	4.49, 21.2	
2.73	0.376	2.73	0.00, 10.0	c	4.11	0.407	2.18	0.00, 10.0	c
8.34	0.102	8.34	5.92, 12.8		3.95	0.111	9.40	6.51, 12.3	
ne dea 100%	—	(none dead at 100%	— —		—	—	(none dead at 100%	— —	
92.2	0.021	92.2	(<0.0), (>100.)	d	3.15	0.022	85.2	48.9 , (>100.)	d
/10 dea 100%	—	(2/10 dead at 100%	— —		—	—	(2/20 dead at 100%	— —	
36.7	0.026	36.7	19.6 , 53.8		3.95	0.019	55.1	40.2 , 70.1	d
/10 dea 100%	—	(2/10 dead at 100%	— —		2.01	0.028	100.	65.0 , (>100.)	d,f
89.5	0.018	89.5	(<0.0), (>100.)		3.19	0.015	100.	16.4 , (>100.)	f
78.8	0.014	78.8	(<0.0), (>100.)	d	3.36	0.023	70.4	56.4 , 84.4	d
16.2	0.023	16.2	(<0.0), (>100.)	d	3.86	0.039	29.1	18.2 , 39.9	d
10 dead 100%	—	(1/10 dead at 100%	— —		—	—	(1/20 dead at 100%	— —	
ne dead 100%	—	(none dead at 100%	— —		—	—	(none dead at 100%	— —	
ne dead 100%	—	(none dead at 100%	— —		—	—	(none dead at 100%	— —	
4.5	0.040	64.5	52.1 , 76.9		2.59	0.034	69.8	58.2 , 81.4	

ethods).

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ignificant.

2

With few exceptions, the degree of toxicity of the effluents from 3A and 6A ranged from relatively low (with high LC_{50} values of 70 percent or greater) to nonexistent (with no deaths at the 100 percent concentration). The exceptions included one replicate using effluent 3A having a low LC_{50} of 36.7 percent and two replicates using effluent 6A from the same day, having LC_{50} values of 41.5 percent and 16.5 percent.

Probit Curves

Figure 4 provides a visual summary of the relationships between fish mortality and effluent concentration. The individual points in the graph were obtained by pooling all data from separate tests of each effluent. Specifically, for each effluent, the percent mortality at a given concentration was calculated by dividing the total number of deaths by the total number of fish tested at that concentration (after standardizing the number of fish per tank to ten so that each test would receive equal weight). After applying this pooling procedure, the new data points obtained were used in probit analyses to determine the five probit curves corresponding to the five different effluents.

The high toxicity level of effluent A is indicated in Figure 4 by its probit curve, which increases sharply to 100 percent mortality at 2.3 percent effluent concentration. Effluent B was less toxic; its probit curve shows a more gradual increase to the 100 percent mortality mark. The mixture of A and B effluents (A+B) was intermediate in toxicity between the individual A and B effluents; its probit curve lies between the individual A and B effluent curves.

The relatively low toxicity levels of effluents 3A and 6A are indicated by the very gradual increases of these two probit curves, which Figure 4 shows to be almost identical. (The data for effluent 6A was pooled after omitting the duplicate runs spiked with RDX.) From the points at which the curves intersect the right vertical axis, it can be seen that an average of 40 to 45 percent mortality resulted from 100 percent concentration of either pilot plant effluent.

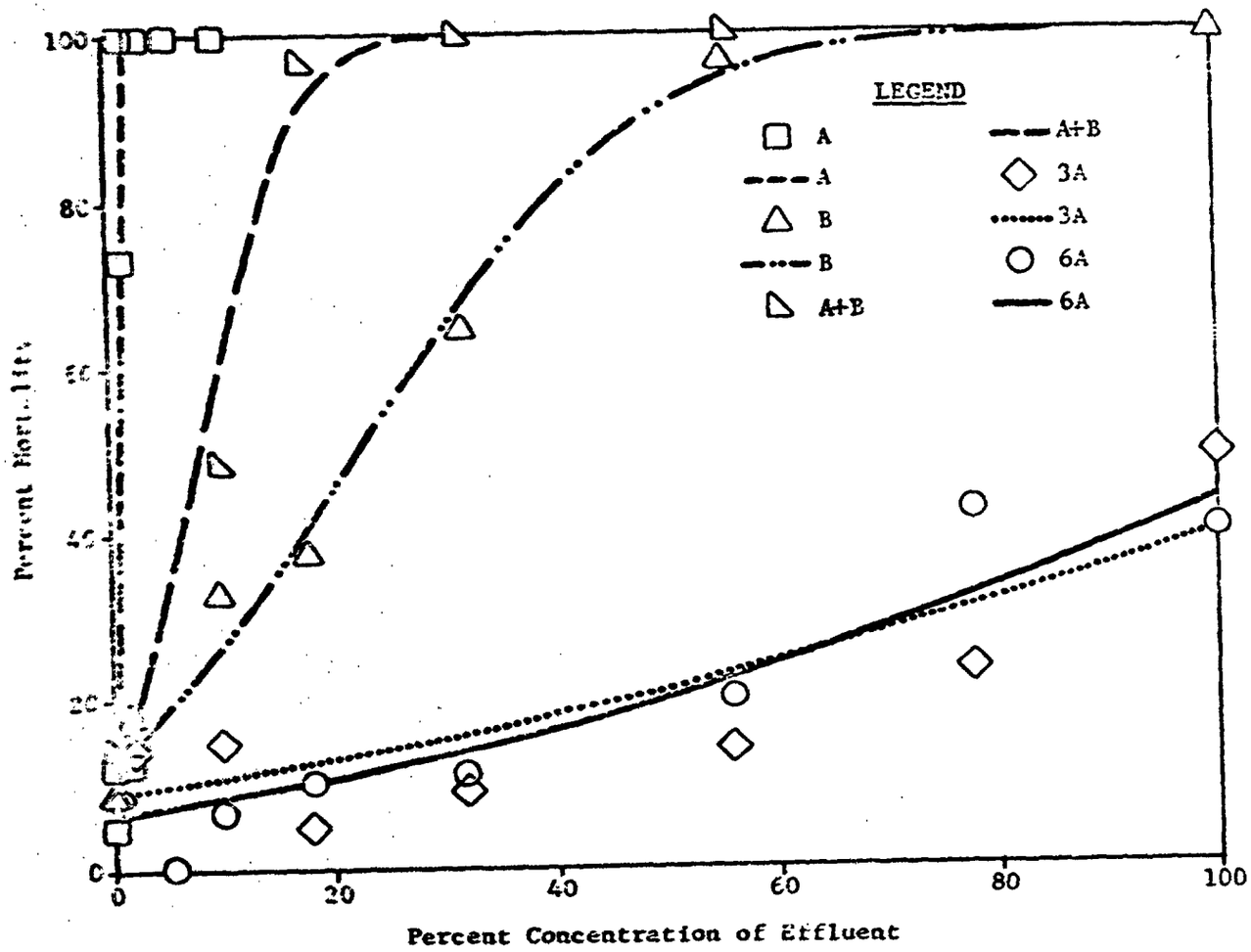

FIGURE 4. FISH MORTALITY VS. EFFLUENT CONCENTRATION OF THE FIVE WASTEWATERS TESTED AT HAAP

The points on the left vertical axis represent the test results from the zero concentrations, or controls. From the individual data points (actual mortalities) as well as the intersection points between the probit curves and the left vertical axis (predicted mortalities), it appears that the natural mortality was about 10 percent in the controls.

Correlations Between LC_{50} Values and Water Quality Data

Table 8 displays the correlation coefficients between LC_{50} values from the bioassay tests and the water chemistry data. A majority of the water quality parameters, notably HMX, showed significant negative correlations with the LC_{50} values. Since a negative correlation means the same thing as an inverse relationship, this shows that high concentrations of HMX are associated with low LC_{50} values and vice versa. The pH levels, on the other hand, were positively correlated with the LC_{50} values, indicating the low pH levels are also associated with low LC_{50} values.

Low LC_{50} values themselves represent increased mortality rates due to increased toxicity. Therefore, Table 8 shows that toxic effects are associated with high levels of HMX, COD, BOD, and so on. A similar inference could not be derived for RDX because the LC_{50} values were not significantly correlated with RDX levels.

The LC_{50} values were not significantly correlated with suspended solids, settleable solids, or D.O., but this may be due to the large numbers of missing data points for these three variables.

Mutagenic Screening

The Ames bacterial mutagenesis assay is extremely simple yet highly efficient in detecting mutagenic compounds. It has been shown to facilitate detection of nanogram quantities of some pure compounds. To be used as a routine screening procedure for environmental specimens, its efficacy is predicated on the basis that a mutagen is either present in extremely high concentration or the compound must have extremely high mutagenic capabilities.

TABLE 8. SIGNIFICANT CORRELATION COEFFICIENTS OF MUNITIONS AND WATER QUALITY PARAMETERS WITH LC_{50} VALUES FROM BIOASSAYS [a]

Parameter	Correlation of Parameters with LC_{50} Values
HMX	-.73
RDX	
COD	-.95
BOD-filtered	-.71
BOD	-.86
pH	+.79
NH_3	-.81
Total Kjeldahl N.	-.80
D.O.	-- --
PO_4	-.78
NO_3	-- --
NO_2	-.78
Suspended solids	--
Settleable solids	-- --
Total solids	-.75

(a) All correlation coefficients given are significant at the 99 percent level or greater. Dashes are given in place of correlations not significant at the 95 percent level.

The mechanism by which the mutagen is detected is by the reverse mutation of the cell to prototroph or the wild form. This wild form is no longer histidine dependent. However, each of the strains used in these investigations has a constant rate of spontaneous reversion. Since the tester strains spontaneously revert, test materials which are only very slightly mutagenic must have a reversion rate sufficiently greater than the spontaneous rate in order to be detected.

The results of the mutagenic bioassay analysis of the wastewater samples are shown in Table 9. The data presented in this table is the relative mutagenicity of the sample analyzed. It is felt that the relative mutagenicity far more easily facilitates detection of mutagens than tables of numbers of revertants per plate. According to this index, a value of 1.0 translates into no mutagenic activity. The numbers of mutants on this plate is equal in number to the number of spontaneous revertants of the tester strain when not exposed to a mutagenic environment. Thus the greater the value of the number beyond 1, the greater the possibility of mutagenic activity. Index values of 2.0 to 3.0 and perhaps slightly higher are best accountable to chance variation and human error, although weak mutagenic activity cannot be overlooked. Values approaching 10.0 and higher are more clearly indicative of activity. It is not possible to establish exact confidence limits from data of this type. Additional testing must be done before rigorous data evaluations can be made.

Inspection of the relative mutagenic activity of the water samples shows that no definite mutagens were detected. One of the tester strains indicated a slight suggestion of the presence of a possible mutagen in Sample 6 (6A effluent collected on June 22, 1976). However, the degree to which the response indicated the possible presence of the mutagen was marginal and was attributed to experimental variability.

The positive control data is presented in Table 10. The data presented shows that the tester strains were capable of detecting the presence of substances which were mutagens in their own right as well as those substances which required microsomal activation for mutagenesis.

TABLE 9. RELATIVE MUTAGENIC ACTIVITIES OF WATER SAMPLES IN SALMONELLA STRAINS TESTED UNDER NON-ACTIVATION AND ACTIVATION CONDITIONS.

Sample Number[a]	µℓ of Sample	Non-Activation					Activation				
		1535	1537	1538	98	100	1535	1537	1538	98	100
		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1	5						0.66	0.62	0.90	0.86	0.72
	10						0.77	0.38	0.60	1.06	0.95
	20						0.44	0.62	0.60	0.95	0.88
	50	0.66	1.50	1.07	1.17	1.55	0.66	0.92	0.60	1.24	1.22
	100						0.55	0.52	0.45	0.77	1.27
2	5						0.55	0.76	1.15	0.84	0.68
	10						0.61	0.76	1.25	0.80	0.57
	20						0.72	0.38	1.45	0.95	0.84
	50	0.94	0.80	0.53	0.79	1.12	1.05	0.77	0.90	0.73	0.82
	100						0.61	0.20	1.05	0.91	0.67
3	5						0.83	0.53	1.00	0.73	0.79
	10						0.61	1.00	1.30	0.82	0.79
	20						0.72	0.53	0.80	0.88	0.88
	50	0.72	1.00	0.84	0.65	1.36	1.05	0.53	0.80	0.73	0.74
	100						1.16	0.61	0.95	0.77	0.85
4	5						1.00	1.07	1.00	0.75	0.81
	10						0.66	0.38	1.50	0.48	0.70
	20						0.55	0.38	0.85	0.80	0.77
	50	0.90	1.25	1.23	0.96	0.94	1.11	0.61	0.55	1.17	0.79
	100						0.72	0.38	1.25	0.55	0.84
5	5						0.83	0.76	1.05	0.82	1.20
	10						1.61	0.76	0.75	0.93	1.22
	20						1.11	0.92	0.60	0.86	1.29
	50	0.88	2.25	1.69	1.68	1.62	0.55	0.76	0.85	0.75	1.40
	100						1.22	0.61	0.95	0.71	1.37

TABLE 9. (Continued)

Sample Number(a)	µl of Sample	Non-Activation					Activation				
		1535	1537	1538	98	100	1535	1537	1538	98	100
		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
6	5						0.76	1.33	1.85	1.03	0.78
	10						0.58	1.67	1.53	0.89	1.02
	50	0.69	0.44	1.13	1.15	0.70	0.53	0.83	1.77	1.13	1.00
	100						0.47	1.50	2.07	1.03	1.02
7	5						0.82	1.33	1.15	1.07	1.38
	10						0.52	0.83	1.23	0.62	0.77
	50	0.77	0.33	1.00	1.00	0.67	0.41	0.50	0.76	1.00	0.96
	100						0.71	1.50	0.92	0.93	1.05

(a) Sample 1 - Area A - June 2, 1976
Sample 2 - Area B - June 2, 1976
Sample 3 - Area A + Area B - June 2, 1976
Sample 4 - Effluent 3A - June 3, 1976
Sample 5 - Effluent 6A - June 3, 1976
Sample 6 - Effluent 6A - June 22, 1976
Sample 7 - Effluent 6A (Spiked) - June 25, 1976

TABLE 10. MUTAGENIC ACTIVITY OF POSITIVE CONTROL COMPOUNDS IN SALMONELLA TESTER STRAINS TESTED IN ACTIVATION AND NON-ACTIVATION CONDITIONS

	Non-Activation		Activation	
TA 1535				
	Sodium Azide (1mg/ml)		2 Aminoanthracine (1mg/ml)	
	100 µl	1836	100 µl	24
	50 µl	1455	50 µl	49
	20 µl	1469	20 µl	357
	10 µl	1326	10 µl	614
	5 µl	1133	5 µl	370
	1 µl	560	1 µl	83
	0 µl	18	0 µl	18
TA 1537				
	Quinacrine HCl (2mg/ml)		2 Aminoanthracine (1mg/ml)	
	100 µl	468	100 µl	111
	50 µl	32	50 µl	114
	20 µl	9	20 µl	706
	10 µl	12	10 µl	604
	5 µl	5	5 µl	182
	1 µl	3	1 µl	42
	0 µl	4	0 µl	13
TA 1538				
	2 Nitrofluorine (1mg/ml)		2 Aminoanthracine (1mg/ml)	
	100 µl	2138	100 µl	1072
	50 µl	1924	50 µl	1637
	20 µl	2816	20 µl	2470
	10 µl	1054	10 µl	2378
	5 µl	624	5 µl	2004
	1 µl	424	1 µl	435
	0 µl	13	0 µl	20
TA 98				
	2 Nitrofluorine (1mg/ml)		2 Aminoanthracine (1mg/ml)	
	100 µl	1494	100 µl	1983
	50 µl	1837	50 µl	2810
	20 µl	2397	20 µl	3794
	10 µl	909	10 µl	2291
	5 µl	749	5 µl	1557
	1 µl	335	1 µl	661
	0 µl	29	0 µl	45
TA 100				
	2 Nitrofluorine (1mg/ml)		2 Aminoanthracine (1mg/ml)	
	100 µl	1596	100 µl	2834
	50 µl	1505	50 µl	2820
	20 µl	1450	20 µl	2864
	10 µl	1154	10 µl	2694
	5 µl	904	5 µl	953
	1 µl	214	1 µl	394
	0 µl	110	0 µl	170

The negative control data on sterility confirmations are not shown. No bacterial contamination was found in the filtered water samples assayed. Likewise, the microsomal activation mix was free of contaminating organisms,

SUMMARY AND CONCLUSIONS

The analyses of the five influent and effluent waters of the HAAP pilot treatment plant showed the concentrations of all munitions determined to be less than 6.0 ppm. Treatment wastewater munitions concentrations ranged generally between <0.05 to 0.7 ppm. (Notable exceptions were HMX levels in the 6A effluent of 2.1 ppm and in the 3A effluent of 2.07 ppm.)

Untreated wastewaters were characterized by high levels of COD, BOD (filtered and unfiltered) NH_3-N, TKN, PO_4, nitrate, nitrite, and solids and low dissolved oxygen concentrations. Area A wastewater had a low pH; Area B wastewater was near 7.0 in pH. Treated wastewaters were generally lower in all parameters except pH which ranged between 7.2 and 8.8. Dissolved oxygen levels remained low.

The correlations presented in Table 6 established a group of munitions constituents and water quality parameters which varied together: HMX, chemical and biological oxygen demand, various forms of nitrogen (NH_3, total Kjeldahl nitrogen, and NO_2), phosphates and total solids. The raw data in Table 5 reflects this clearly: high values of all these parameters were found in the Area A effluent samples, low values were found in the 3A and 6A treatment samples, and intermediate values were found in the Area B and the combined areas A and B effluent samples. Table 8 shows that high toxicity levels are strongly associated with these same munitions and water quality variables.

The 3A and 6A treatment effluents appeared to be almost identical both in terms of their chemical analyses and their bioassay test responses. The calculated LC_{50}'s for treated water's 3A and 6A were both greater than 100 percent; the expected mortalities at the 100 percent concentration of each effluent were 40 percent and 43 percent, respectively.

It is notable that RDX was not significantly correlated with any water quality parameters (except total solids) nor with toxicity levels.

The most consistently high toxicity levels resulted from the Area A effluent, which had RDX concentrations ranging from .43 to 1.90 ppm; yet a substantially lower toxicity level resulted from the 6A effluent spiked with RDX, which had a higher RDX concentration of 5.17 ppm. Also, the LC_{50} values resulting from the 6A effluent tests spiked with RDX were about the same as, or even lower than, those resulting from some of the unspiked 6A effluent tests (none of which contained detectable concentrations of RDX). These findings indicate that RDX levels were not high enough in any of the effluent samples to cause clearcut increases in fish mortality. A possible synergistic effect from the presence of several compounds may have occurred in the wastewater.

The multiple internal correlations among the munitions constituents and water quality parameters make it difficult to determine exactly which factors were responsible for the extreme toxicity of the Area A effluent. Fish mortalities may have been caused by the high levels of each of several parameters (for example NH_3 concentrations were reported within ranges shown to be toxic to fish [Becker and Thatcher, 1973]) or by the interaction effects resulting from the combination of them. Also, the pH may have been low enough to increase the effects of these factors. The pH of the undiluted Area A effluent samples was about 3.5. Assuming that the Holston River water had a pH of 7, these values can be converted to percent hydrogen ion concentrations and combined in a weighted average to give an estimate of the pH of the solutions used in the bioassay tests. For instance, the pH of the 1 percent solution of Area A effluent would have been about 5.5, according to these calculations.)

In general, significant Pearson's correlations among variables indicate a strong degree of association but cannot in themselves be used to establish cause-and-effect relationships. Therefore, another statistical technique was used in an effort to determine whether the presence of the munitions compounds had an effect on toxicity levels separate from the effects of other potentially causative factors. Partial correlation coefficients were calculated between the LC_{50} values and the munitions compounds by controlling each of these other factors. A partial correlation coefficient is similar to an ordinary Pearson's r correlation, except that it controls, or holds constant, a third variable. This method of

correlation is appropriate when it is believed that the third variable is somehow interfering with the Pearson's correlation between the first two variables, by either creating a "spurious" correlation where none actually exists, or by masking a true relationship which does exist.

Table 11 presents partial correlation coefficients of LC_{50} values with HMX and RDX, computed by controlling each of four variables separately: COD, BOD, NH_3, and total Kjeldahl nitrogen. (LC_{50} values over 100 percent were omitted from the calculations.) All the partial correlations between HMX and LC_{50} were significant indicating that HMX does have an influence upon toxicity levels separate from the effects of COD, BOD, and so on. RDX and the LC_{50} values were not significantly correlated when the controlled variable was either COD or BOD; significant correlation occurred when either NH_3 or TKN was controlled. This latter correlation indicates that RDX may have been found to be significantly related to toxicity levels if the various forms of nitrogen had been at a fixed level throughout all the effluent samples.

TABLE 11. PARTIAL CORRELATIONS BETWEEN MUNITIONS CONCENTRATIONS AND LC_{50} VALUES (a)

Controlled Variable	Partial Correlation Between HMX and LC_{50}	Partial Correlation Between RDX and LC_{50}
COD	-.51	--
BOD	-.61	--
NH_3	-.67	-.58
Total Kjeldahl-N	-.69	-.56

(a) Correlation coefficients given are significant at the 99 percent level or greater. Dashes are given in place of correlations not significant at the 95 percent level.

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